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Mitochondria in peroxisome-deficient hepatocytes exhibit impaired respiration, depleted DNA, and PGC-1α independent proliferation

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ABSTRACT

The tight interrelationship between peroxisomes and mitochondria is illustrated by their cooperation in lipid metabolism, antiviral innate immunity and shared use of proteins executing organellar fission. In addition, we previously reported that disruption of peroxisome biogenesis in hepatocytes severely impacts on mitochondrial integrity, primarily damaging the inner membrane. Here we investigated the molecular impairments of the dysfunctional mitochondria in hepatocyte selective *Pex5* knockout mice. First, by using blue native electrophoresis and in-gel activity stainings we showed that the respiratory complexes were differentially affected with reduction of complexes I and III and incomplete assembly of complex V, whereas complexes II and IV were normally active. This resulted in impaired oxygen consumption in cultured $Pex5^{-/-}$ hepatocytes. Second, mitochondrial DNA was depleted causing an imbalance in the expression of mitochondrial- and nuclear-encoded subunits of the respiratory chain complexes. Third, mitochondrial membranes showed increased permeability and fluidity despite reduced content of the polyunsaturated fatty acid docosahexaenoic acid. Fourth, the affected mitochondria in peroxisome deficient hepatocytes displayed increased oxidative stress. Acute deletion of PEX5 in vivo using adeno-Cre virus phenocopied these effects, indicating that mitochondrial perturbations closely follow the loss of functional peroxisomes in time. Likely to compensate for the functional impairments, the volume of the mitochondrial compartment was increased several folds. This was not driven by PGC-1α but mediated by activation of PPARα, possibly through *c-myc* overexpression. In conclusion, loss of peroxisomal metabolism in hepatocytes perturbs the mitochondrial inner membrane, depletes mitochondrial DNA and causes mitochondrial biogenesis independent of PGC-1α.

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1. Introduction

Over the years, it has become clear that peroxisomes and mitochondria have a strong interrelation as they exhibit complementary activities, share proteins and communicate with each other. Metabolically, both organelles are involved and cooperate in the degradation of fatty acids [1,2]. Very long and branched chain fatty acids are shortened in peroxisomes, followed by transfer to the mitochondria for further breakdown. Some of the enzymes participating in β-oxidation such as alpha-methylacyl-CoA racemase are targeted to both organelles [3]. Moreover, both peroxisomes and mitochondria contribute to the detoxification of oxygen radicals generated in cellular processes [4]. In addition, it was recently shown that induction of oxidative stress in peroxisomes affects the mitochondrial redox balance via unresolved mechanisms [5]. These organelles also depend on the same proteins for the execution of their fission process such as Dlp1, Fis1 and Mff1 [6–9]. A defect in Dlp1 can even lead to a combined peroxisomal–mitochondrial disorder [10]. Furthermore, other proteins such as MAVS that are involved in antiviral signaling were shown to reside both on the peroxisomal and outer mitochondrial membrane [11]. A surprising finding was the existence of an intracellular transport route between mitochondria and peroxisomes whereby mitochondria derived vesicles can incorporate selected cargo and fuse with peroxisomes [12–14].

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Another illustration of the tight link between these organelles is that mitochondrial abnormalities arise when peroxisomes are dysfunctional. In a few early reports on Zellweger syndrome, the most severe peroxisome biogenesis disorder, mitochondrial abnormalities were documented in hepatocytes. These changes were not studied in detail but included structural alterations at the inner mitochondrial membrane and a reduction in the activities of several complexes of the respiratory chain [15–19]. Importantly, in different Zellweger syndrome mouse models generated by disruption of *Pex5* [20,21], *Pex2* [22], or *Pex13* [23], swollen mitochondria with sparse and abnormally shaped cristae were found in hepatocytes.

Mitochondrial anomalies were also recapitulated in a liver-selective *Pex5* knockout mouse model (*L-Pex5*$^{-/-}$) that survives into adulthood [24]. These mice develop microvesicular steatosis, hepatomegaly, and fibrosis. The reduced abundance of cristae was accompanied by impaired activity of complexes I, III and V as determined by spectrophotometry and by an energetic deficit leading to the activation of the cellular energy sensor AMPK [25]. In contrast, mitochondrial matrix enzymes were more active in mutant hepatocytes as shown for citrate synthase [24] and mitochondrial β-oxidation [26].

At present, the mechanisms through which peroxisomal inactivity detrimentally impacts on mitochondrial structure and function are unresolved. In order to gain insight into these intriguing organellar interactions, we have investigated in more detail the properties of the aberrant mitochondria in peroxisome deficient hepatocytes, including the expression of the respiratory complexes, redox state, lipid composition, fluidity and permeability of the membranes, and DNA content. Furthermore, the time course of mitochondrial impairments was investigated by acutely deleting PEX5 from hepatocytes. Finally, we investigated whether and how compensatory mitochondrial proliferation is activated.

2. Materials and methods

2.1. Mouse breeding

L-Pex5$^{-/-}$ mice were generated by crossing Albumin-Cre and *Pex5*$^{FL/FL}$ mice as previously described [24]. All experiments were performed on 8–15-week-old *Pex5*$^{FL/FL}$, considered as controls and littermate *L-Pex5*$^{-/-}$ mice, unless otherwise stated. *Pex5*$^{FL/FL}$ mice were also brought into a PPARα deficient background (provided by Prof. F. Gonzalez [27]). In some experiments *Pex5* was acutely inactivated in hepatocytes by intravenous administration of adenovirus encoding Cre-recombinase (3.10^9 pfu) or control adenovirus in *Pex5*$^{FL/FL}$ *PPARα*$^{-/-}$ or *Pex5*$^{FL/FL}$ *PPARα*$^{+/+}$ as previously described [25]. Mice were maintained on a 12-hour light/12-hour dark schedule and were fed standard rodent food chow and water ad libitum. All animal experiments were approved by the Institutional Animal Ethical Committee of the University of Leuven.

2.2. Analysis of mitochondria

2.2.1. Purification of mitochondria

L-Pex5$^{-/-}$ and wild-type mice were killed by cervical dislocation and livers were rapidly removed, washed in ice-cold homogenization medium (HM) (0.25 M sucrose, 5 mM Mops, 1 mM EDTA, 0.1% (v/v) ethanol, pH 7.2), minced and homogenized in a Potter homogenizer with a motor-driven pestle (1200 rpm). Cellular debris was pelleted by centrifugation of the homogenate at 770 *g* for 10 min. The post-nuclear supernatant (PNS) was spun for 10 min at 2330 *g* and the pellet was washed once to obtain a fraction enriched in mitochondria. For some experiments, this fraction, corresponding to 1.5 g of liver, was layered on top of a Percoll solution (40% (w/v) Percoll, 0.22 M sucrose, 1 mM Mops, 1 mM EDTA, 0.1% (v/v) ethanol, pH7.2) and centrifuged for 1 h at 4 °C at 34,000 *g* (Beckmann ultracentrifuge) as described before [28] but using only 18 ml of Percoll solution. Subsequently, 500 μl fractions were collected starting from the bottom. The distribution of the organelles was monitored by measurement of marker enzymes [mitochondria (glutamate dehydrogenase, GDH); lysosomes (acid phosphatase); endoplasmic reticulum (glucose-6-phosphatase)]. Mitochondrial enzymes were recovered in fractions 2–22 of the 38 fractions that were collected. The 5 fractions containing the highest activity of the mitochondrial marker enzyme GDH were combined, diluted 20 times in HM, and centrifuged (10 min, 4 °C, 2330 *g*). Based on Western blot and EM analysis these fractions primarily contained mitochondria and were only contaminated to a minor extent with peroxisomes, ER or lysosomes. The purified mitochondrial pellet was resuspended in 1 ml of HM and used immediately or stored at −80 °C for further analysis.

2.2.2. Quantification of mitochondria

Purified mitochondria were counted on a FACSCanto flow cytometer (BD) using BD Trucount™ tubes.

2.2.3. Electron microscopy on isolated mitochondria

The Percoll-purified mitochondrial pellet was resuspended in a cell free system (CFS) buffer (10 mM HEPES, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 5 mM pyruvate, 0.5 mM EGTA, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, pH 7.4), centrifuged for 3 min, and fixed in freshly prepared 4% (w/w) glutaraldehyde and 8% (w/v) BSA in CFS buffer for 30 min on ice. The pellet was washed with sodium cacodylate buffer supplemented with 1% (w/v) $CaCl_2$ and prepared for electron microscopy.

2.2.4. Blue native gel electrophoresis of the oxidative phosphorylation (OXPHOS) complexes

Blue native gel electrophoresis followed by in-gel activity staining was performed as described [29] on isolated liver mitochondria. Samples of controls and knockouts were loaded in duplicate (60 μg/lane). In the first set of lanes, activity staining of complexes I, III and IV was performed, and the second set of lanes was used for the activity staining of complexes II and V. The bands resulting from complexes I, II, III and IV activities were scanned in transmission mode. The complex V band, which was seen as a white precipitate, was scanned in 'reflexion' mode using a dark background for better visualization [29].

2.2.5. Mitochondrial transmembrane potential

The membrane potential of purified mitochondria was analyzed essentially as in [30]. Briefly, purified mitochondrial suspensions were incubated in medium (110 mM KCl, 75 mM mannitol, 20 mM MOPS, 10 mM glutamate, 1 mM malate, 1 mM EGTA, albumin 1 mg/ml, pH 7.2) for 1 min at room temperature in the dark with JC-1 (1.5 μg/mg protein) in a final volume of 0.5 ml. Suspensions were analyzed immediately using a FACSVantage SE flow cytometer (BD) equipped with a single 488 argon laser. The filter in front of the fluorescence 1 (FL1) photo multiplier (PMT) transmits at 530 nm and has a bandwidth of 30 nm, and the filter used in the FL2 channel transmits at 585 and has a bandwidth of 42 nm. To analyze mitochondria stained with JC-1, the PMT value of the detector in both FL1 FL2 PMT was set at 480 V; FL1–FL2 compensation was 1.6%, and FL2–FL1 compensation was 30%.

2.2.6. Measurement of fluorescence anisotropy

Mitochondrial membrane fluidity was evaluated by fluorescence anisotropy of the mitochondria-bound dyes 1,6-diphenyl 1,3,5-hexatriene (DPH) and trimethylammonium (TMA)-DPH according to [31]. Freshly prepared mitochondrial suspensions (0.5 mg/ml) were incubated in Standard Incubation Medium (SIM; 200 mM sucrose, 10 mM Tris–HCl, 1 mM KH_2PO_4, 10 μM EGTA, 3 μM rotenone, 0.5 μg oligomycine and 5 mM succinate; pH 7.4) either with DPH (50 μM; diluted from a stock solution of 20 mM (prepared in tetrahydrofurane) with 10 mM Tris–HCl pH 7.4, 150 mM KCl, 1 mM EDTA) or TMA-DPH (3 μM) for 30 min at 37 °C, under continuous stirring. Steady-state fluorescence polarization was measured in a Hitachi spectrofluorometer (ex 366 nm: em 425 nm). The results are expressed as anisotropy units (r), where $r = (I_0/I_{90}) / (I_0 + 2I_{90})$. I_0 and I_{90} represent the intensities of light when polarizers were in parallel or in perpendicular orientations, respectively.

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